

20030310174

AD-P008 770



IMMUNOCHEMICAL AND BIOCHEMICAL ANALYSIS OF  
ADDUCTS TO DNA AND PROTEINS AFTER EXPOSURE TO  
SULFUR MUSTARD

G.P. van der Schans, L.P.A. de Jong\*, R.H. Mars-Groenendijk,  
H.P. Benschop\* and R.A. Baan,

TNO Medical Biological, P.O.Box 5815 2280HV Rijswijk, and  
\*Prins Maurits Laboratories, P.O. Box 45, 2280 AA Rijswijk,  
The Netherlands.



94-07932

ABSTRACT

The confirmed use of chemical agents in the Iran-Iraq conflict and the threat of their use in the recent Gulf War have stressed the need of reliable methods for detection of poisoning with such agents. We have chosen to define exposure to sulfur mustard (HD), based on immunochemical analysis of adducts of HD to DNA and proteins. These adducts are agent-specific and may be stable *in vivo* for several days or even for months.

The detection methods described here are applied to blood samples to establish exposure and -if possible- to estimate internal dose. A major HD-DNA adduct has been identified as N7-(2'-hydroxyethylthioethyl)-guanine. HD-DNA adducts in nucleated cells of blood treated with  $\geq 2 \mu\text{M}$  HD are detectable with monoclonal antibodies raised against this monoadduct. Recently, the immunochemical assay has been modified to an immunoslot-assay which is more reproducible because of its simplicity. Furthermore, it has at least the same detection limit as observed in the ELISA used previously. Application of immunofluorescence microscopy allows detection down to  $0.3 \mu\text{M}$  HD (see next paper of this proceedings).

The same HD-DNA adduct can also be determined by means of HPLC with electrochemical detection. This method will be used for calibration of the immunochemical assays.

Analogous to HD-DNA adducts, reaction products with blood proteins may be used to establish HD exposure. Since it was found that the amino-terminal valine in the  $\alpha$ -chain of hemoglobin is alkylated after HD-exposure, the amino-terminal heptapeptide from this protein was synthesized and alkylated with HD. The development of an immunochemical assay for this amino-terminal HD-adduct to hemoglobin has been initiated. We found that cysteine is the amino acid most susceptible to alkylation by HD. Therefore, a tetrapeptide hapten containing the cysteine HD-adduct and haptens consisting of HD-adducts attached to peptide sequences of hemoglobin are also being prepared for future use in immunizations.

This work is supported in part by the US Army Medical Research and Development Command under Contracts DAMD17-88-2-8022 and DAMD17-92-V-2005.

# Best Available Copy

## COMPONENT PART NOTICE

THIS PAPER IS A COMPONENT PART OF THE FOLLOWING COMPILATION REPORT:

TITLE: Proceedings of the Medical Defense Bioscience Review (1993)

Held in Baltimore, Maryland on 10-13 May 1993. Volume 1.


TO ORDER THE COMPLETE COMPILATION REPORT, USE AD-A275 667

THE COMPONENT PART IS PROVIDED HERE TO ALLOW USERS ACCESS TO INDIVIDUALLY AUTHORED SECTIONS OF PROCEEDING, ANNALS, SYMPOSIA, ETC. HOWEVER, THE COMPONENT SHOULD BE CONSIDERED WITHIN THE CONTEXT OF THE OVERALL COMPILATION REPORT AND NOT AS A STAND-ALONE TECHNICAL REPORT.

THE FOLLOWING COMPONENT PART NUMBERS COMPRISE THE COMPILATION REPORT:

AD#: P008 752 thru P008 794 AD#: \_\_\_\_\_  
AD#: \_\_\_\_\_ AD#: \_\_\_\_\_  
AD#: \_\_\_\_\_ AD#: \_\_\_\_\_

**DTIC**  
**ELECTE**  
**MAR 15 1994**  
**S F D**

Accession For	
NTIS GRA&I	<input checked="" type="checkbox"/>
DTIC TAB	<input checked="" type="checkbox"/>
Unannounced	<input checked="" type="checkbox"/>
Justification	
By _____	
Distribution /	
Availability Codes	
Dist	Avail
A-1	

This document has been approved  
for public release and sale its  
distribution is unlimited.

## INTRODUCTION

The recent wars in the Gulf Area have demonstrated that the use of CW-agents is more than an imaginary threat. Experience with the casualties of these conflicts has made it clear that an urgent need exists for reliable methods to establish - on the basis of patient-specified indicators - the nature and dosage of CW-agents to which casualties have been exposed. Such methods may involve the detection of the agent or its reaction products in biological material; they should be specific and also sensitive, and fast when treatment depends on

the results of the assay. A problem is posed by the fact that biological samples often become available only days or even weeks after exposure, when the agent or its decomposition products have been eliminated.

Therefore, methods to establish exposure to HD, preferably should not be based on the detection of these rapidly eliminated substances. Instead, we develop analytical procedures that are aimed at the detection of adducts of HD to DNA and protein, which are expected to remain present in the body for a considerable period of time.

Our method of choice is the immunochemical detection of HD adducts for the following reasons:

- 1) Adducts are agent-specific alkylation products
- 2) These adducts may have a life span of several days or even months.
- 3) The procedure is meant primarily for application on blood and skin samples, to verify exposure and to establish internal dose. In order to achieve high sensitivity and specificity, immunochemical detection methods provide high sensitivity and specificity: for DNA, such techniques have been shown often to provide a selectivity of 1 modified nucleotide in up to  $10^8$  unmodified bases in DNA and a sensitivity in the femtomole ( $10^{-15}$  mol) range.
- 4) It is expected that immunochemical detection techniques can be applied "on site", for example in a field hospital.

The strategy we followed to develop these immunochemical methods is:

- 1) Characterization of adducts
- 2) Selection and synthesis of the immunogen(s)
- 3) Preparation of antibodies
- 4) Development of immunochemical detection methods
- 5) Calibration of the method with independent biochemical detection methods (e.g., HPLC-ECD, i.e., High Performance Liquid Chromatography with electrochemical detection and mass spectrometric analysis).
- 6) Detection of damage in biological samples.

## METHODS

### *Human white blood cells*

Venous blood was collected from volunteers in 10-ml evacuated glass tubes containing 15 mg EDTA. Blood was treated with 0.3-40  $\mu$ M HD (60 min at 37°C) and the white blood cells (WBC) were isolated after lysis of the erythrocytes.

### *Isolation of DNA*

The WBC were lysed overnight with 1% sodium dodecyl sulfate and proteinase K (0.5 mg/ml). Next day, the DNA was purified by extraction with phenol and precipitation with

ethanol and dissolved in PBS (0.14 M NaCl, 2.6 mM KCl, 8.1 mM Na<sub>2</sub>PO<sub>4</sub> and 15 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). The DNA, still double-stranded, was sonicated and directly thereafter tested in the immunochemical assays and with HPLC-ECD (see below).

#### *Polyclonal rabbit antiserum against HD-treated DNA*

Rabbits were immunized twice with double-stranded calf thymus DNA, that had been treated with 1 mM HD (30 min, 37°C) and complexed to methylated bovine serum albumin, and once with single-stranded calf thymus DNA (prepared by heating of double-stranded calf thymus DNA for 5 min at 100°C) treated with 10 µM HD (30 min, 37°C) and complexed to methylated bovine serum albumin.

#### *Monoclonal antibodies against N7-G-HD monoadduct in DNA*

Mice were immunized with N7-(2'-hydroxyethylthioethyl)-guanosine-5'-monophosphate (GMP-7-HD) coupled with 1-(3-dimethyl-aminopropyl)-3-ethylcarbodiimide to Keyhole Limpet hemocyanin (KLH). Hybrid cells were obtained after fusion of the spleen cells with plasmacytoma cells. Specific antibody production was assayed in a direct ELISA with control DNA and HD-modified DNA as immobilized antigens and with goat-anti-mouse-IgG-alkaline phosphatase conjugate to detect antibody binding. Several clones produced antibodies specific for HD-modified DNA. The antibodies of one of these, 2F8, have been used in the immunoassays.

#### *Enzyme-linked immunosorbent assays (ELISA)*

Enzyme-linked immunosorbent assays have been described in detail elsewhere (1). Briefly, the wells of microtiter plates were coated with single-stranded HD-treated calf thymus DNA, to which the adduct-specific antibodies can bind. The antibody molecules bound to the coating DNA are detected with second antibodies (goat-anti-rabbit or goat-anti-mouse-IgG) that carry a detection enzyme. The optimal conditions for the ELISA system appeared to be: concentration of HD for coating-ss-DNA: 10 µM; amount of ss-DNA applied in microtiter plates for coating: 50 ng/200-µl well; dilution of polyclonal serum: 1/40,000. The supernatants of the monoclonal antibodies were diluted 1/1000 in PBS containing 0.05% Tween 20 and 0.1% gelatin. The purified monoclonal antibodies were diluted in the same solution; the extent of dilution was chosen such that 30% of the highest detectable level was reached in 2 h of incubation for the sample without inhibitor DNA.

To determine the amount of adducts in DNA, the ELISA was carried out in the competitive mode. The sample-DNA is first made single-stranded, without destroying the ring-closed structure of the imidazole moiety of N7-guanine-HD in the DNA, by heating for 25 min at 52°C in the presence of 4% formamide, 0.2% formaldehyde and 0.01 M tris and 1 mM EDTA, and mixed at various dilutions with a certain amount of antiserum or monoclonal antibodies. The amount of binding of antibodies to the DNA is proportional to the amount of adducts present. The extent of residual unbound antibodies is determined in a normal ELISA.

#### *Immunoslotblot assay*

In the immunoslotblot assay (2) the single-stranded HD-treated DNA is first adsorbed to a cellulose nitrate filter (pore size 0.1 µm; Schleicher and Schuell). Then the filter is treated with milkpowder (Campina, Eindhoven, The Netherlands; less than 1% fat) in order to prevent a-specific antibody binding. After washing the filters are treated with N7-G-HD monoadduct-specific monoclonal (2F8) antibody (1:500 dilution of culture supernatant)

followed by treatment with a second antibody (RAM-PO, diluted 1:1000) directed against the first one and conjugated to the enzyme horse-radish peroxidase. The activity of the enzyme is determined by addition of a substrate (luminol) which is converted into a chemiluminescent product in the presence of  $H_2O_2$ . The amount of chemiluminescence produced during a certain time period is detected by exposure of a photographic film.

#### *HPLC with electrochemical detection.*

The ring-closed N7-G-HD monoadduct has been analysed, according to the method described by Park *et al* (3), as the free base adduct (N7-G-HD) by HPLC with electrochemical detection in human blood exposed to various concentrations of HD. Briefly, N7-G-HD is released from DNA by neutral hydrolysis, after which DNA is removed by precipitation with hydrochloric acid followed by centrifugation. Then, the supernatant is neutralized and N7-G-HD is detected by reversed phase HPLC combined with electrochemical detection. Synthetic N7-G-HD is used as a standard for calibration.

The system consisted of two pumps (acting out-of-phase to reduce pump-pulse effects on the EC-signal, each delivering 50% of the eluent; ABI400, Ramsey, NJ, USA); a pulse damper (ABI); a reversed-phase column (Chromospher C-18, Chrompack, Middelburg, The Netherlands, 100x3 mm, particle size 5  $\mu$ m); an amperometric detector cell with a glassy carbon working-electrode and an Ag/AgCl reference electrode (Antec, Leiden, The Netherlands). The eluents used contained 1 mM n-octyl sodium sulfate, 9% methanol and 25 mM  $H_3PO_4$  adjusted to pH 6.0 with KOH. Flow rates of 0.5 ml/min were used. The oxidation potential of the detector was 1.345 V and the background current varied from 20-50 nA.

## RESULTS AND DISCUSSION

#### *Competitive ELISA*

Four adducts to DNA were identified, via HPLC analysis of the products obtained after degradation of the DNA (1). The major DNA damage induced by HD appeared to be the N7-guanine monoadduct. It constitutes about 60% of all adducts to DNA in white blood cells (WBC) in human blood exposed to HD.

On the basis of the polyclonal and monoclonal antibodies described above immunochemical tests (ELISA) were developed. The sensitivity of a competitive ELISA based on the polyclonal serum was estimated with samples of single-stranded calf thymus DNA containing known amounts of the N7-G-HD adduct. Figure 1 shows the inhibition curves in the competitive ELISA for single-stranded calf thymus DNA alkylated with 0, 0.01, 0.1, 1 and 10  $\mu$ M HD, respectively. (The adductlevel after exposure to 0.01  $\mu$ M HD amounts to about 1 N7-G-HD monoadduct per  $2 \times 10^6$  nucleotides). Evidently, the untreated DNA does not give any inhibition, whereas an increasing degree of alkylation gives increasing inhibition.

The assay has been also applied to DNA of WBC in human blood exposed to HD *in vitro* (Figure 2). Also in this case inhibition could be observed.

The lower detection levels for DNA exposed under different circumstances are summarized in Table 1. It should be noted that with immunofluorescence microscopy the lower detection limit of HD exposure of whole blood is about 0.3  $\mu$ M (see our next paper in this proceedings).

With the monoclonal antibodies directed against the N7-G-HD we could obtain the same sensitivity in the immunochemical assay as with the rabbit antiserum.

Table 1. The lower detection limit of HD exposure of ss- and ds-DNA and whole blood.

	detection level	N7-G-HD adduct/guanine
ss-ct-DNA:	10 nM HD	1:1,290,000
ds-ct-DNA	10 nM HD	1: 750,000
human blood	2 $\mu$ M HD	1: 100,000

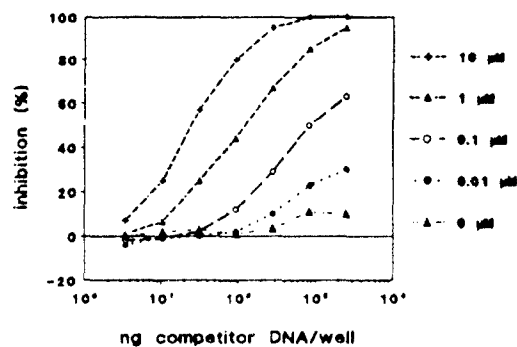


Fig. 1. Competitive ELISA with rabbit antiserum and single-stranded calf-thymus DNA as competitor. The DNA was treated with HD at the concentrations indicated in the figure and then tested in the competitive ELISA.

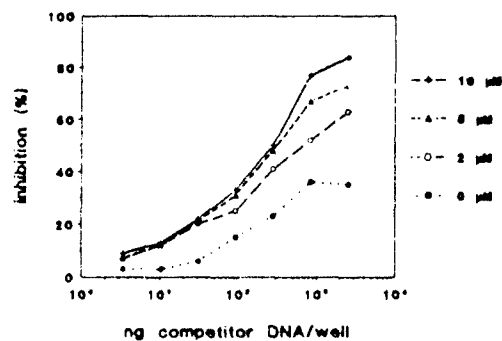


Fig. 2. Competitive ELISA with rabbit serum and DNA isolated from human white blood cells as competitor. Whole blood was treated with HD at the concentrations indicated in the figure. After isolation of the white cells, DNA was isolated by phenol extraction and ethanol precipitation and made single-stranded by heating as indicated under "Methods".

#### *Immunoslotblot assay*

A variant of the enzyme-linked immunosorbent assay described above is the so-called immunoslotblot assay. In the latter assay, the single-stranded HD-treated DNA is first adsorbed to a cellulose nitrate filter, followed by treatment with (i) monoadduct-specific monoclonal antibody (ii) a second antibody directed against the first one and conjugated to the enzyme horse-radish peroxidase, and (iii) addition of a substrate. In this way it appeared possible, after blotting of 0.1-1  $\mu$ g DNA per slot to detect exposure of the DNA to 0.01  $\mu$ M HD. This is the same detection limit as in the competitive ELISA, but because of its simplicity, the immunoslotblot assay is easier to reproduce. Following exposure of human blood to 10  $\mu$ M HD, N7-G-HD could be easily detected in this way. The lower detection limit has still to be determined. Experiments are in progress to improve the sensitivity of the assay, particularly by improving the denaturation conditions of double-stranded DNA.

#### *HPLC with electrochemical detection.*

In the earlier experiments  $^{35}$ S-labelled HD was used for calibration, by determination of the amount of radioactivity in the adduct peaks after HPLC analysis of the  $^{35}$ S-products released from treated DNA (1). Presently, we are able to quantitate the adduct peaks directly via HPLC with electrochemical detection of the N7-guanine adduct according to the procedure described above.

In Figure 3 chromatograms are shown with the characteristic N7-G-HD peak at 5.21 min retention time.

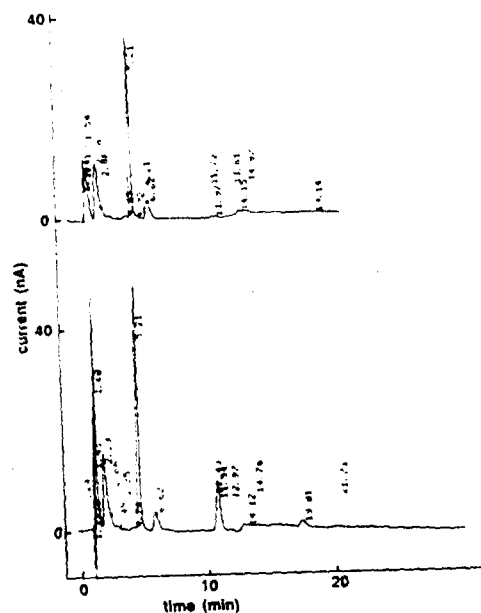


Fig. 3. HPLC-ECD-chromatograms of the marker (N7-G-HD; upper panel) and of the hydrolysate of calf thymus DNA exposed to HD (lower panel). The peak at 5.21 min represents the N7-G-HD monoadduct.

There is a linear increase of the peak height with the amount of adduct between 0.05 and 10 pmol of adduct (Figure 4). With this method the absolute adduct levels could be established in samples of single-stranded calf thymus DNA that had been exposed to 0.1-100  $\mu$ M HD (Figure 5).

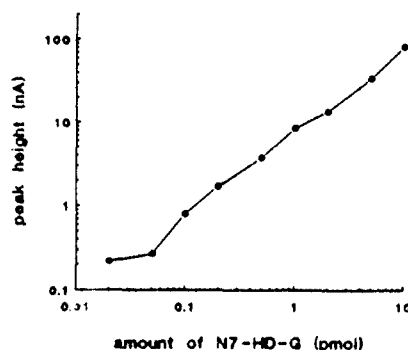


Figure 4. The peak height of the N7-G-HD peak in the HPLC-ECD-chromatogram as a function of the amount injected.

As can be derived from Fig 4, the lower detection limit amounts to about 50 fmol of N7-G-HD. N7-G-HD could be detected in calf thymus DNA exposed to HD at concentrations of  $\text{HD} \geq 0.1 \mu\text{M}$  (Fig. 5). The adduct level appeared to be about 4 times higher than that observed previously under the same conditions.

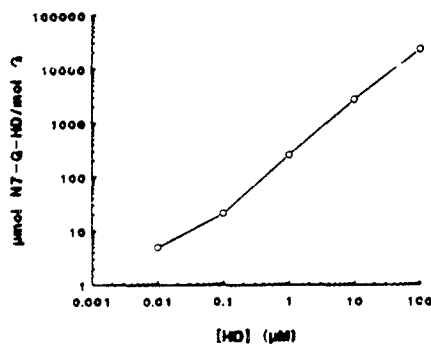


Figure 5. Amount of N7-G-HD in ss-ct-DNA after exposure to HD at the concentrations indicated. The amount of N7-G-HD was detected by means of HPLC-ECD.



The N7-G-HD monoadduct could also be detected electrochemically in DNA of WBC of human blood exposed to HD. These data are summarized in Table 2.

Table 2. Degree of alkylation with HD in calf thymus DNA and DNA from white blood cells measured with HPLC and electrochemical detection.

Sample	Concentration HD ( $\mu$ M)	Ratio N7-G-HD/unmodified guanine ( $\times 10^6$ )
ss-ct-DNA	0.1	21
	1	255
	10	2693
	100	22600
blood	1	0
	10	48
	100	356

The adduct level in DNA in white blood cells appeared to be about 60 times lower than in calf thymus DNA exposed in solution to HD at the same concentration and about 3 times lower than observed previously in DNA in human blood exposed to  $^{35}$ S-labelled HD. We do not have explanations for these discrepancies with the earlier data. Possibly, the rate of degradation of HD in aqueous solution may vary considerably.

In parallel samples the adducts will be analysed by the immunochemical assays, in order to validate the latter.

#### *Antibodies against protein-adducts of HD*

There are good arguments to expect advantages in the use of protein adducts for establishing exposure to HD. The living cell is well equipped with enzyme systems that remove DNA lesions within days. Such systems are not known with respect to cellular proteins. In the body, therefore, protein adducts will be longer-lived than those to DNA, provided that the involved protein does not have a rapid turn-over. Moreover, in blood ca. 1000 x larger amounts of HD are bound to protein than to DNA, as we observed in experiments with [ $^{35}$ S]-HD in human blood (1), although less is bound per unit weight. However, many groups in proteins may react with HD. Therefore, the choice of the most profitable group for detection purposes is difficult due to lack of sufficient data.

In a systematic approach to the synthesis of protein haptens, we have prepared simple model derivatives of those amino acids that can be alkylated by HD in their side chains. The  $\alpha$ -amino and  $\alpha$ -carboxylic groups of the amino acids were acylated and amidated, respectively. Their primary reaction products with HD were identified by means of LC-thermospray MS. The free carboxylic acid functions of glutamic and aspartic acid were found to be alkylated, while cysteine and methionine react at the sulfur atom. Both ring nitrogens of histidine can be alkylated.

A protein abundantly present in blood, with a known, long half life of several months, is hemoglobin. The free aminogroup of the N-terminal valine in the  $\alpha$ -chain of human

hemoglobin is rather exposed in the native protein structure and is alkylated *in vivo* by various alkylating agents. This valine can be obtained as part of the N-terminal heptapeptide that can be easily isolated after release with trypsin. The alkylated heptapeptide was found to contain ca 6% of the total radioactivity bound to hemoglobin after exposure to [<sup>35</sup>S]-HD. We synthesized the heptapeptide and alkylated its valine with HD. The N-alkylated heptapeptide was bound to a carrier protein and injected into mice. Several IgM-monoclonal antibodies were obtained having affinity for HD adducts with hemoglobin. Since IgM antibodies are not very suitable for application in the competitive ELISA tests, our efforts to obtain IgG-antibodies against adducts of HD to the N-terminal valine are continued. In addition, we found that cysteine is the amino acid most susceptible to alkylation by HD. Therefore, a tetrapeptide hapten containing the cysteine HD-adduct and also other haptens consisting of HD-adducts attached to peptide sequences of hemoglobin are also being prepared for future use in immunizations.

### CONCLUSIONS

With antibodies directed against the most abundant HD adduct to DNA, the N7-guanine monoadduct, ELISA's were developed which detect this adduct in ss- and ds-DNA after exposure to 10 nM HD.

In blood, exposure to 1-2  $\mu$ M HD is detectable with these assays.

Several lines of investigation are pursued to obtain antibodies directed against the HD-modified N-terminal heptapeptide of hemoglobin.

The immunochemical detection methods for mustard gas adducts in blood and skin samples are useful for:

- diagnosis of systemic and local exposure to HD, as well as for triage and dosimetry in casualties,
- research on the efficacy of topical skin protection and skin contamination,
- research on healing of skin damage, allowing to follow the repair of various cell types in the skin.

### Acknowledgements

The authors thank Ms. Eline Scheper for carrying out the HPLC-ECD assays.

### REFERENCES

1. Benshop, H.P. Verification, dosimetry and biomonitoring of sulfur mustard exposure via immunochemical detection of mustard gas adducts to DNA and proteins. Final report for grant No DAMD17-88-Z-8022; December 1991. AD-A240 471.
2. Nehls, P., Adamkiewicz, J., and Rajewski, M.F. (1984). Immuno-slot-blot: a highly sensitive immunoassay for the quantitation of carcinogen-modified nucleosides in DNA. J. Cancer Res. Clin. Oncol. 109, 23-29.
3. Park, J.-W, Cundy, K.C., and Ames, B.N. (1989). Detection of DNA adducts by high-performance liquid chromatography with electrochemical detection. Carcinogenesis, 10, 827-832.